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Umbilical Cord Mesenchymal Stem Cells Increase Expansion of Cord Blood Natural Killer Cells

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ABSTRACT

Natural killer (NK) cell-mediated cytotoxicity can control leukemia relapse while protecting patients from graft-versus-host disease (GVHD) after allogeneic stem cell transplant. Cord blood (CB) is rich in NK cell progenitors with similar properties of proliferation and cytotoxicity as adult blood NK cells. Hence, it is attractive to expand and potentially utilize these cells for adoptive immunotherapy. In this study, CB mononuclear cells were CD3-depleted by immunomagnetic microbead selection to remove T cells. This $CD3^{dep}$ CB-MNC fraction was then plated for *ex vivo* expansion, with or without a feeder layer of irradiated umbilical cord mesenchymal stem cells (UC-MSC), with or without cytokines that have been shown to be critical for NK expansion: IL-2, IL-15, IL-3, and FLT-3L. At an average of 2 weeks of culture, there was significantly higher expansion (64.7 ± 8.4-fold) of $CD56^+/CD3^-$ NK cells in the presence of the UC-MSC feeder layer and cytokines compared to controls (no increase with feeder layer only and 6.4 ± 1.5-fold increase with cytokines only, $P < .05$). Contact between $CD3^{dep}$ CB-MNC cells and UC-MSC augmented NK expansion. The combination of all 4 cytokines was superior to IL-2 alone or 2 cytokines combinations: mean 64.7 ± 8.4-fold expansion with 4 cytokines combination versus IL-2 alone, IL-2 + FLT-3L, IL-2 + IL-15 or IL-2 + IL-3 (12.2 ± 2.0, 14.4 ± 2.4, 10.4 ± 4.1, 25.2 ± 8.1 respectively). We also observed that only fresh $CD3^{dep}$ CB-MNC preparations could be expanded reliably, whereas frozen and thawed $CD3^{dep}$ CB-MNC cells did not expand consistently (mean fold increase 6.5 ± 3.2). Cytotoxicity of expanded NK cells was compared with NK cells from fresh and overnight IL-2 activated $CD3^{dep}$ CB-MNC. Whereas fresh cells displayed no discernible killing, strong cytotoxicity against K562, Raji, REH, and SUP-B15 cells lines was noted after overnight activation in IL-2. Cytotoxicity of expanded NK cells against Raji, REH, and SUP-B15 was lower, which, however, correlated with a predominant expansion of $CD56^+/CD16^-$ cells known to have less cytolytic activity than $CD56^+/CD16^+$. To test the transfection efficiency in NK cells, fresh or expanded $CD3^{dep}$ CB-MNC cells were electroporated with either DNA or mRNA constructs for GFP. DNA had a low transfection efficiency (<10%), whereas the one for mRNA reached 52%, but at the cost of significant cell death. Our results suggest that CB NK cell progenitors can be expanded to obtain large numbers by using an irradiated feeder of UC-MSC. They maintain an elevated cytotoxic profile, and may be genetically manipulated—all characteristics that make them suitable for cellular therapies.

KEY WORDS

Cord blood • NK cell • Cytotoxicity • Mesenchymal stem cells

INTRODUCTION

Natural killer (NK) cells represent a subpopulation of lymphocytes that express CD56 and CD16 (FcγRIII) but lack lineage specific antigens for T cells (CD3), B cells (CD19), or monocytes (CD14) [1-4]. They mediate antibody-dependent cellular cytotoxicity as well as "spontaneous" killing of infected or transformed cells through release of perforin and granzyme from cytotoxic granules. NK cells are also able of secreting immunoregulatory cytokines, particularly interferon (IFN)-γ, TNF-α, and GM-CSF. Recent preclinical and clinical data suggest that NK cells may play a pivotal

role in the outcome of HLA-haploidentical stem cell transplantation by reducing leukemia relapse and protecting recipients from graft-versus-host disease (GVHD) [5,6]. Further studies in patients with advanced AML have suggested that infusion of CD3-depleted MHC 1-haplotype mismatched mononuclear cells enriched for NK cells, can induce remissions in about 20% of patients with chemotherapy resistant AML [7].

Umbilical cord blood (CB) is rich in hematopoietic stem cells, and has become an alternative source for stem cell transplantation for hematologic malignancies [8,9]. As a stem cell source for transplant it offers several advantages over unrelated marrow or blood stem cells such as ease of availability, tolerance to greater HLA mismatch, and potentially lower incidence of GVHD. Yet its success is limited by cell dose and lack of donor lymphocyte infusions to eliminate post-transplant residual disease or treat relapse. Several approaches are under investigation to improve the CB transplant outcome, including coinfusion of multiple units, *ex vivo* expansion of CB stem cells, reduced intensity conditioning (RIC), and development of adoptive immunotherapies [9,10].

Adult NK cells are derived from $CD34^+$/HLA-DR^+ human progenitor cells, which differentiate into NK progenitors. Certain cytokines provide a synergistic culture environment for the differentiation of NK progenitor cells such as FLT-3L, IL-3, and IL-15 [11-13]. Committed immature NK cells progressively acquire CD56 and CD16 expression as well as killer cell immunoglobulin-like receptors (KIRs), natural cytotoxicity receptors (NCRs), and CD94-NKG2 receptors. CB contains a higher percentage of NK progenitor cells than adult blood, which can mature into NK cells with significant cytotoxicity [14-16].

Mesenchymal stem cells (MSC) can be obtained from various tissue sources, and are capable of differentiating into tissues of mesenchymal and endodermal origin as well as support hematopoiesis and inhibit T cell proliferation [17]. The umbilical cord contains a jelly-like matrix (Wharton's Jelly), rich in pluripotent mesenchymal stem cells (UC-MSC), which has been recently characterized as a novel source of MSC [18-22]. These cells produce significant amounts of cytokines, and we hypothesized that a feeder layer of irradiated UC-MSC would effectively support *ex vivo* expansion of CB-NK cells through cytokine stimulation. The objective of this study was to determine whether an UC-MSC-based expansion system for CD3-depleted CB-MNC ($CD3^{dep}$ CB-MNC) cells could lead to clinically relevant numbers of NK cells that can be considered for cellular immunotherapy. We sought to optimize expansion conditions including use of different cytokine combinations and source of feeder cells (from same or allogeneic cords), and contact versus no contact with UC-MSC feeder cells. Our results show that the feeder layer system results in a >60-fold expansion of NK cells, which remain functional and could be considered for cell therapies.

MATERIALS AND METHODS

Collection and Preparation of CB and UC-MSC

CB samples and umbilical cords were obtained from normal full-term vaginal or Caesarian deliveries after informed consent. The study was approved by the institutional review board of Tufts Medical Center (Boston, MA). The umbilical cord was rinsed with PBS and cut into small pieces (0.5-1 cm) and placed into 6-well plates. UC-MSC were isolated as described previously [19] and expanded in culture flasks with 20% FBS/RPMI 1640 (Cambrex, Walkersville, MD) supplemented with antibiotics (Penicillin 100 IU/mL, Streptomycin 100 μg/mL, Amphotericin B 0.25 μg/mL from Gibco Invitrogen (Carlsbad, CA), and Ciprofloxacin 10 μg/mL from Mediatech (Herndon, VA). CB was collected in citrate phosphate dextrose (CPD) sterile blood-pack unit (Baxter, Deerfield, IL) and processed within 24 hours after delivery. CB-MNC were isolated by density gradient centrifugation using Ficoll-Hypaque Plus (Amersham Biosciences, Piscataway, NJ) and depleted of $CD3^+$ lymphocytes by immunomagnetic bead selection using the MidiMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The final elute, designated here as $CD3^{dep}$ CB-MNC, was used to initiate NK expansion and consistently contained <1% $CD3^+$ cells. Cells were either freshly cultured or cryopreserved with 10% DMSO in FBS at −80°C and thawed.

NK Expansion Cultures

UC-MSC (1×10^4 cells/well) were plated in 24-well plates with 20% FBS/RPMI 1640 overnight, gamma-irradiated with 3500 cGy, and incubated for 2-4 hours before being used as a feeder layer. $CD3^{dep}$ CB-MNC were plated with or without the feeder layer of UC-MSC either from the same (autologous) or from an unrelated (allogeneic) cord donor, with or without different combinations of the following cytokines: IL-2 (1000 IU/mL; Chiron, Emeryville, CA), IL-15 (10 ng/mL), IL-3 (10 ng/mL), and FLT-3L (10 ng/mL; R&D Systems, Minneapolis, MN). To determine optimum condition, expansions were performed with different $CD3^{dep}$ CB-MNC:UC-MSC ratios. To determine whether contact between UC-MSC and $CD3^{dep}$ CB-MNC cells was necessary, expansions with direct feeder contact at 1:1 ratio were compared with those where cells were separated by a 1.0-μm pore PET membrane cell culture insert (Becton Dickinson, Franklin Lakes, NJ). Cells were harvested at an average of 16 ± 2 days, counted with

trypan blue exclusion, and analyzed by flow cytometry. NK expansion was calculated as follows.

Fold expansion = [(final expanded MNC number) × (final % of $CD56^+/CD3^-$)]/[(initial MNC number) × (initial % of $CD56^+/CD3^-$)].

Cell Lines

Human leukemia cell lines K562 (CML in blast crisis), Raji (human Burkitt's lymphoma), REH, and SUP-B15 (both acute B-precursor ALL), were obtained from American Type Culture Collection (ATCC, Rockville, MD). These cell lines were maintained in suspension cultures using 20% FBS/RPMI 1640 as described above.

Phenotyping of Surface Antigens, and Flow Cytometry

Phycoerythrin (PE)-conjugated anti-CD56, allophycocyanin (APC)-conjugated anti-CD117, fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD19, and anti-CD14 were obtained from BD Pharmingen (San Diego, CA). APC-conjugated antibodies against CD16, CD158a (KIR2DL1/DS1), CD158e (KIR3DL1), CD158i (KIR2DS4), CD335 (NKp46), CD336 (NKp44), CD337 (NKp30), and CD314 (NKG2D) were obtained from Miltenyi Biotech (Auburn, CA). The expression of these markers on $CD3^{dep}$ CB-MNC before and after expansions was analyzed by flow cytometry on a Cyan™ flow cytometer (Dako, Carpinteria, CA) using Summit v4.3 software.

Cytotoxicity Assay

Cytotoxic activity of NK cells from fresh, overnight IL-2 activated, or 2 weeks expanded $CD3^{dep}$ CB-MNC was determined by a flow cytometry-based assay [23]. Briefly, target cells were prestained with the green fluorescent membrane dye PKH67-GL (Sigma-Aldrich, St. Louis, MO), and effector cells were mixed with target cells in 96-well V-bottom plates at various effector-to-target (E:T) ratios, in 20% FBS/RPMI 1640. After incubation at 37°C for 4 hours, the cell mixtures were centrifuged at 1700 rpm for 5 minutes and stained with Propidium Iodide (PI), 10 μg/mL (Sigma-Aldrich). Dead target cells were identified as PKH67-GL and PI double positives. Target cells and effector cells were also stained separately with PI to assess spontaneous cell lysis. Percentage of target cell killing was calculated as follow:

% Killing = (% total target lysis) − (% spontaneous target lysis).

The NK to target ratios were calclated using the actual number of $CD56^+/CD3^-$ NK cells within the $CD3^{dep}$ CB-MNC population used as an effector.

Transfection of Expanded NK Cells with Reporter Genes

Fresh or expanded $CD3^{dep}$ CB-MNC were transfected by electroporation with plasmid pCMV-GFP (gift from Dr. Lidija Covic, Tufts Medical Center, Boston, MA), or with GFP mRNA obtained by *in vitro* transcription (T7 mMessage mMachine kit, Ambion Applied Biosystems, Austin, TX) of a pXT7-GFP template plasmid (gift from Pr. Sergei Sokol, Mount Sinai School of Medicine, New York), according to the manufacturer's instructions. Two different electroporation methods were tested: Genepulser II electroporator (Biorad, Hercules, CA) using different conditions of voltage and capacitance, and the Nucleofactor transfection system (Amaxa Biosystems, Cologne, Germany) using a human NK cell Nucleofactor Kit (Amaxa) according to the manufacturer's instructions. GFP fluorescence and PI staining in the $CD56^+/CD3^-$ NK population were determined 24 hours after electroporation using flow cytometry.

Statistical Analysis

Data are presented as mean ± SEM values. For statistical analysis, Student's *t*-test was used. Groups compared were considered to be significantly different if $P < .05$.

RESULTS

UC-MSC Support NK Cell Expansion

Fresh $CD3^{dep}$ CB-MNC cocultured with UC-MSC for 2 weeks, at a $CD3^{dep}$ CB-MNC to a UC-MSC ratio of 10:1 (with all 4 cytokines) showed an expansion of 12.8 ± 3.0-fold in total MNC number. Culture with cytokines in the absence of UC-MSC resulted in MNC expansion of 4.1 ± 0.8-fold ($P = .024$), and cocultured with UC-MSC without cytokines did not support any MNC expansion at all (0.2 ± 0.1-fold, $P = .004$) (Table 1). Cocultures using different $CD3^{dep}$ CB-MNC to UC-MSC ratios showed inferior expansion (data not shown). In cocultures with UC-MSC and cytokines the proportion of $CD56^+/CD3^-$ cells increased from 9.2 ± 1.4% in fresh $CD3^{dep}$ CB-MNC to 48.6 ± 2.7% after 2 weeks, which corresponded to a 64.7 ± 8.4-fold expansion of NK cells. NK expansion in culture with UC-MSC feeder layer only or with cytokines only was 0.1 ± 0.1-fold and 6.4 ± 1.5-fold, respectively (Figure 1A). No $CD3^+$ or $CD19^+$ cells were detected after 2 weeks under any of these conditions. Expansion of frozen and thawed $CD3^{dep}$ CB-MNC samples under the same culture conditions was poor (6.5 ± 3.2-fold increase of NK) and inconsistent, with high variability between cord samples. In contrast, using fresh or cryopreserved UC-MSC from autologous or allogeneic sources as a feeder layer did not show any difference with respect to their ability to support NK cells expansion (data not

Table 1. *CD3 Depleted CB-MNC Expansion in the Presence of Different Combinations of Cytokines, with or without a Feeder Layer of UC-MSC, at a $CD3^{dep}$ CB-MNC:UC-MSC Ratio of 10:1*

Categories	Mean Fold MNC Expansion	*P*	Initial NK%	Final NK%	n
All CYT w/UC-MSC	12.8 ± 3.0	—	9.2 ± 1.4	48.6 ± 2.7	8
IL-2+FLT-3L w/UC-MSC	3.5 ± 0.7	0.017	10.8 ± 2.2	48.7 ± 2.5	4
IL-2+IL-15 w/UC-MSC	2.1 ± 0.3	0.009	10.8 ± 2.0	48.5 ± 12.4	4
IL-2+IL-3 w/UC-MSC	11.6 ± 4.9	0.831	10.8 ± 2.0	26.7 ± 6.4	5
IL-2 w/UC-MSC	1.9 ± 0.3	0.009	10.8 ± 2.0	63.4 ± 5.7	5
All CYT w/o UC-MSC	4.1 ± 0.8	0.024	9.2 ± 1.4	13.8 ± 2.3	8
No CYT w/UC-MSC	0.2 ± 0.1	0.004	10.8 ± 2.0	2.7 ± 1.1	5
No CYT w/o UC-MSC	0.1 ± 0.1	0.004	10.8 ± 2.0	2.5 ± 2.2	5

NK percentage was determined by the proportion of $CD56^+/CD3^-$ cells in the population, *P* values were calculated compared with all cytokines w/UC-MSC.

shown). Therefore, only data obtained with fresh $CD3^{dep}$ CB-MNC and allogeneic UC-MSC are reported here. Of note is the observation that the UC-MSC feeder layer started to disappear within the first week of culture, with no UC-MSC left after the 2-week culture period.

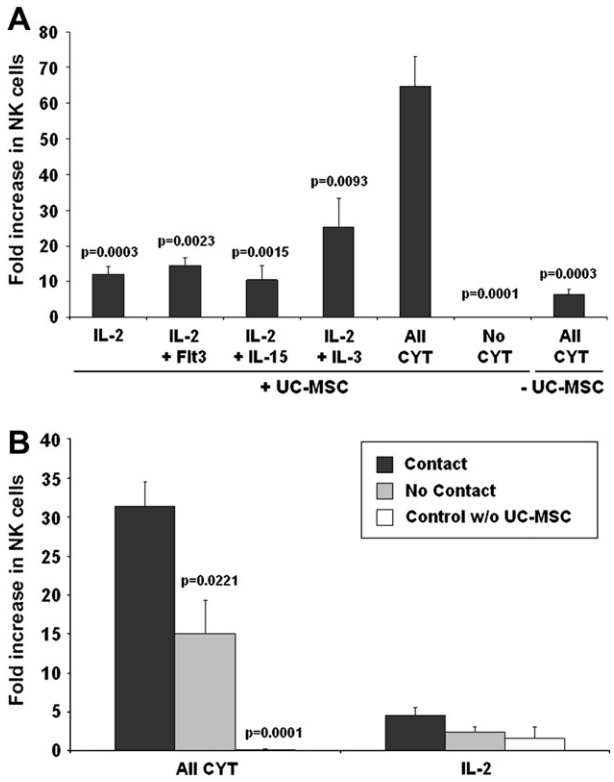

Figure 1. Optimization of NK cell expansion. (A) Comparison of expansion of NK cells with or without a UC-MSC feeder layer ($CD3^{dep}$ CB-MNC:UC-MSC ratio of 10:1) and in the presence of different cytokine combinations (all CYT = IL-2, IL-3, IL-15, and FLT-3L). Expansions are reported as fold increases in $CD56^+/CD3^-$ NK cells. (B) Comparison of expansion of NK cells with or without contact with a UC-MSC feeder layer. Cultures were performed in 6-well plates at a $CD3^{dep}$ CB-MNC:UC-MSC ratio of 1:1, in the presence of all 4 cytokines or IL-2 alone. Controls were without UC-MSC feeder layer.

Cytokine and Contact Requirements for Optimum NK Expansion

The combination of 4 cytokines (IL-2, IL-3, IL-15, FLT-3L) was compared to IL-2 alone and 2 cytokine combinations described below, all expanded on a feeder layer of irradiated UC-MSC. Mean fold NK expansion with the 4-cytokine combination (see above) was significantly greater than with IL-2 alone (12.2 ± 2.0), IL-2 + FLT-3L (14.4 ± 2.4), IL-2 + IL-15 (10.4 ± 4.1), or IL-2 + IL-3 (25.2 ± 8.1) (Figure 1A). IL-2 alone promoted higher NK percentage in the cultured population (63.4 ± 5.7%) but did not promote significant MNC proliferation. In contrast, combination of IL-2 and IL-3 promoted MNC proliferation to a similar extent as the combination of 4 cytokines but favored cell types other than NK (26.7 ± 6.4%) (Table 1).

Expansion of NK cells was significantly greater when $CD3^{dep}$ CB-MNC cells were directly plated onto irradiated UC-MSC cells (a ratio of 1:1) compared to cell insert cultures where the feeder layer was placed at the bottom of the plates and $CD3^{dep}$ CB-MNC cells were seeded on the cell insert membrane (Figure 1B). In the presence of the combinations of 4 cytokines (IL-2, FLT-3L, IL-15, and IL-3), mean fold NK expansion at 2 weeks was 31.3 ± 3.3 with contact and 15.0 ± 4.2 without contact.

Surface Antigen Expression of Fresh and Expanded NK Cells

NK cells were analyzed by flow cytometry using 3-color staining for expression of CD16, CD117, KIR2DL1/DS1, KIR3DL1, KIR2DS4, NKp46, NKp44, NKp30, and NKG2D within the $CD56^+CD3^-$ NK cells population, before and after 2 weeks expansion, with and without UC-MSC feeder cells (Figure 2). Intermediate and late NK precursors have been recently characterized as $CD56^+/CD3^-/CD117^+$ cells [24]. Fresh $CD3^{dep}$ CB-MNC contained 3.3 ± 0.7% of these precursors, whereas $CD3^{dep}$ CB-MNC expanded with or without UC-MSC

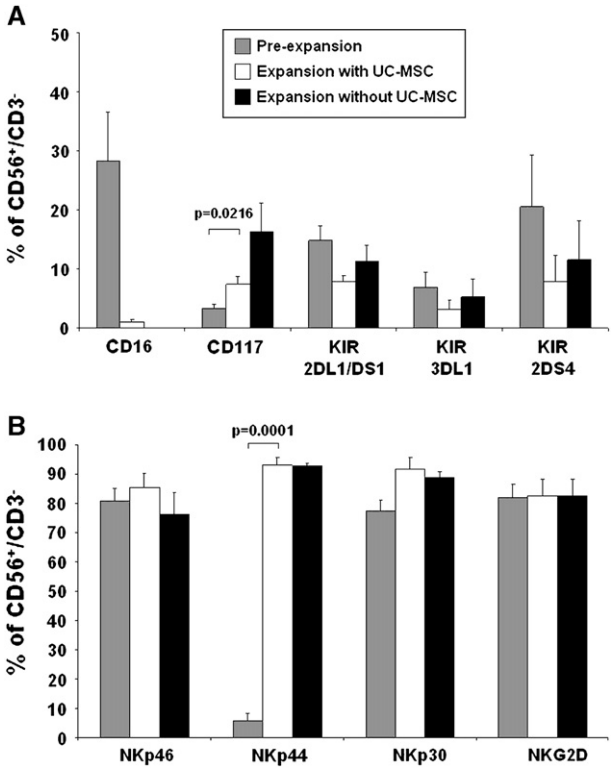

Figure 2. Comparison of expression of NK receptors before and after expansion (A, B). Percentages of $CD16^+$, $CD117^+$, KIR2DL1/DS1$^+$, KIR3DL1$^+$, KIR2DS4$^+$, NKp46$^+$, NKp44$^+$, NKp30$^+$, and NKG2D$^+$ among $CD56^+/CD3^-$ cells were determined before (gray) and after expansion, with a feeder layer (white) at a $CD3^{dep}$ CB-MNC:UC-MSC ratio of 10:1 or without a feeder layer (black).

contained 7.4 ± 1.3% (P = .0216) and 16.2 ± 4.8%, respectively.

The initial proportion of $CD56^{bright}$ cells within the NK population was 6.9 ± 1.4% in fresh $CD3^{dep}$ CB-MNC and significantly increased to 76.8 ± 9.4% and 71.5 ± 7.5% in $CD3^{dep}$ CB-MNC expanded with or without UC-MSC feeder, respectively. Although NK cells from fresh $CD3^{dep}$ CB-MNC expressed some CD16 (28.4 ± 8.3%), expanded NK cells did not express CD16. The percentage of NK cells expressing NKp46, NKp30, and NKG2D did not change significantly before and after 2 weeks in culture, with or without feeder (Figure 2B). Conversely, expression of NKp44 was greatly increased after expansion (93.0 ± 2.6% and 92.8 ± 1.0% with and without MSC, respectively, compared to 5.5 ± 2.8% initially, P = .0001).

Cytotoxicity of Fresh, Activated, and Expanded NK Cells

Fresh $CD3^{dep}$ CB-MNC were tested for NK cells cytotoxic activity against K562, Raji, REH, and SUP-B15 cell lines. Nonactivated $CD3^{dep}$ CB-MNC cells did not efficiently lyse any of these targets (Figure 3A). However, overnight incubation in IL-2 (1000 U/mL) resulted in a significant increase in cytotoxicity against all 4 cell lines (Figure 3B). $CD3^{dep}$ CB-MNC cells expanded on UC-MSC feeders showed strong killing of K562 but consistently lower killing of REH, Raji, and SUP-B15 than observed for $CD3^{dep}$ CB-MNC cells after overnight incubation in IL-2 (Figure 3C). $CD3^{dep}$ CB-MNC cells expanded without UC-MSC feeders (cytokines only) showed very limited cytotoxic activity against K562 and none against the other cell lines (Figure 3D).

Transfection of Expanded NK cells with Reporter Genes

One of the major thrusts in cellular therapies is to be able to engineer cells such that they can be targeted to specific tumor antigens. To test transfection efficiency of NK cells, we used plasmid GFP DNA and mRNA with different transfection protocols and conditions. As summarized in Table 2, transfection efficiency of NK cells from fresh or expanded $CD3^{dep}$ CB-MNC using a Biorad electroporator with a variety of different conditions was no more than 4.5% for GFP DNA (up to 10% for GFP mRNA). The Nucleofactor system from Amaxa, that provides an NK cell-customized transfection medium, also gave poor transfection yields for GFP DNA (9.0 ± 2%) but significantly better transfection for GFP mRNA (52 ± 18%). However, reproducibility of the transfection was inconsistent, and cell mortality caused by both transfection protocols was significant (74.0 ± 12.5% for Amaxa, 90.0 ± 7% for Biorad).

DISCUSSION

In this study, we aimed to define and optimize conditions for *ex vivo* expansion of NK cells from CB, which are potential tools for adoptive immunotherapy. NK cells are emerging as an alternative to T cells, particularly because they can be given across MHC barriers [2,4-6]. Because lymphocyte infusion from the original stem cell donor is not an option for CB transplant recipients and the number of NK cells is limited in a given CB unit, we developed a method that allows to expand NK cells and progenitors to cell numbers that could be sufficient to treat patients posttransplant. Beyond the treatment for patients after transplant, expanded NK cells could also be administered to cancer patients, and could potentially be more therapeutically efficacious if they are engineered to be targeted to individual cancers, that is, by introducing a chimeric antigen receptor (CAR) recognizing tumor antigens [23,25-27]. Earlier studies had already shown that CB contains a fair number of NK cell progenitors that can be identified by expression of surface antigens CD34 and HLA-DR and that combination of the cytokines FLT-3L, IL-2, IL-3, and IL-15 results in some expansion of CB progenitor cells in culture [11-13].

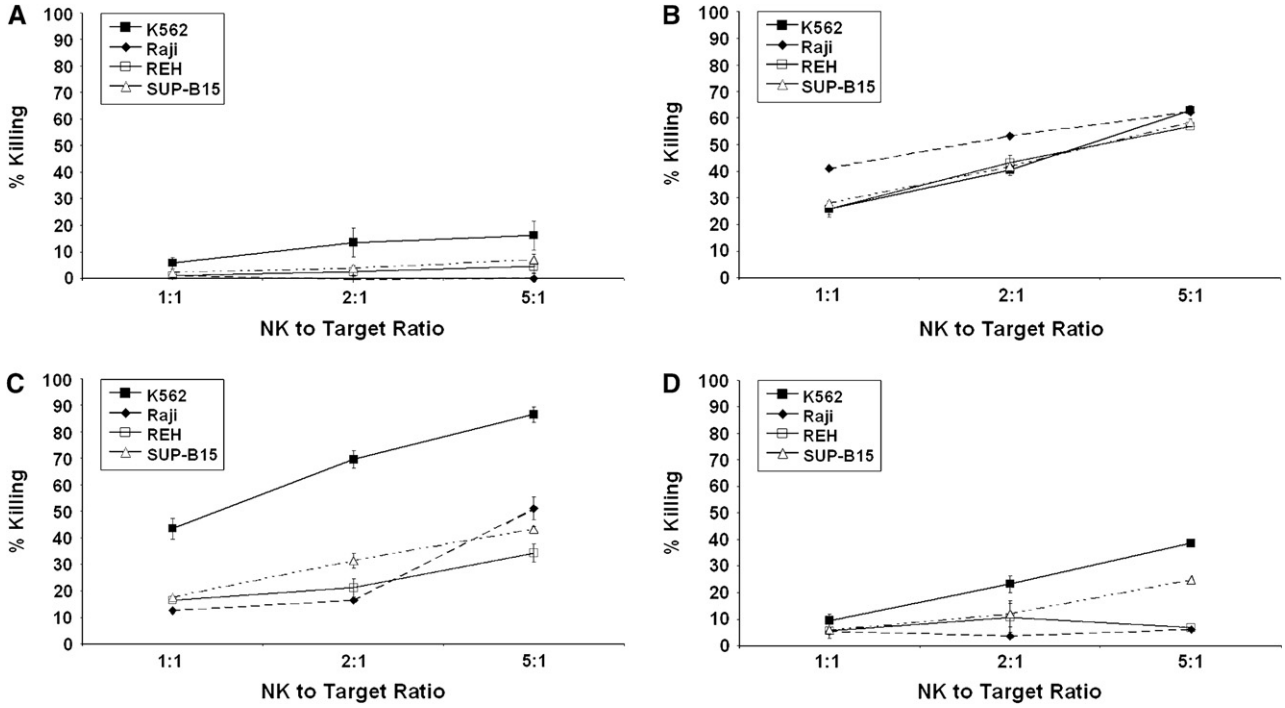

Figure 3. Comparison of the cytotoxic properties of fresh, activated, and expanded NK cells. $CD3^{dep}$ CB-MNC were tested for NK cells cytotoxicity against K562 (black square), Raji (black diamond), REH (white square), and SUP-B15 (white triangle) either on the same day (A), after overnight incubation with IL-2 (1000 IU/mL) (B), and after expansion with (C) or without (D) a UC-MSC feeder layer in the presence of all 4 cytokines. The indicated ratios represent effector (=$CD56^+/CD3^-$ NK) to target.

Similarly, use of irradiated lymphocytes or EBV transfected B cells as feeder layers can result in augmented expansion, which likely occurs through production of lymphokines by the feeder cells [28]. However, this approach is not suitable for clinical applications, as unwanted cells remain in the "gemisch" that potentially can cause GVHD or infections.

We pursued here a different approach to augment expansion of NK cells using a feeder layer of allogeneic irradiated MSC derived from the Wharton's jelly of the umbilical cord. To prevent a GVHD reaction in recipients of an allogeneic NK cell product or overgrowth of T cells in the expansion cultures, $CD3^+$ lymphocytes were removed from CB-MNC by immunomagnetic separation. The remaining cells ($CD3^{dep}$ CB-MNC) were then placed on the UC-MSC feeder supplemented with the combination of cytokines mentioned before. Under these conditions we were able to obtain on average 65-fold expansion of NK cells after 2 weeks in culture and without T cell contamination. It is quite possible that some of the cells that remain after CD3 depletion of CB-MNC (such as monocytes) actually contribute to the expansion of NK cells. This assumption is supported by observations by Gada et al. [29] that highly enriched $CD56^+$ cells from CB-MNC showed inferior *ex vivo* expansion (only 4.5-fold) compared to CB-MNC that were CD3-depleted only (14-fold). Both cell fractions were cultured with IL-2 and IL-15 for 2 weeks.

Of clinical relevance is our observation that frozen $CD3^{dep}$ CB-MNC cells inconsistently expanded, and if so, generally at a much lower frequency. Activated

Table 2. *Comparison of Viability and Expression of GFP in Expanded NK cells When Using Different Conditions of Electroporation and Different Vectors*

Nucleic Acid Type	Device	Conditions	% GFP	Range (%)	% PI^+	Range (%)
cDNA	Amaxa	Prg U-01	9.3 ± 1.9	7 to 13	73.5 ± 12.5	61 to 86
mRNA	Amaxa	Prg U-01	52.5 ± 18.4	0 to 80	74.0 ± 12.5	50 to 92
cDNA	Biorad	300V 150 μF	4.5 ± 4.5	0 to 9	N/A	/
cDNA	Biorad	400V 400 μF	2.0	N/A	N/A	/
mRNA	Biorad	250V 300 μF	0.3 ± 0.3	0 to 0.5	90.0 ± 7.0	83 to 97
mRNA	Biorad	300V 150 μF	10.0 ± 10.0	0 to 30	89.5 ± 5.3	83 to 96
mRNA	Biorad	300V 300 μF	0.4 ± 0.4	0 to 0.8	89.5 ± 7.5	82 to 97

GFP % in the table was determined in the viable (PI negative) cell population.
N/A = not available.

NK cells have been shown to die upon freezing, at least when using standard cryopreservation conditions [30], and it is conceivable that NK progenitor cells in CB are variably sensitive to cryopreservation.

Our data using cell culture inserts, that separate $CD3^{dep}$ CB-MNC cells from UC-MSC, suggest that contact between these 2 cell populations is advantageous for optimized expansion of NK cells. At this time it remains speculative which surface molecules on UC-MSC are responsible for this effect. Of note is that even without contact there was better expansion of NK cells in cultures that contained UC-MSC than in cultures that did not contain any feeder layer. This indicates that UC-MSC produce molecules/cytokines that are supportive of NK progenitor cell expansion. MSC produce a host of cytokines, and it appears that the cytokine profile of "earlier" MSC (such as UC-MSC) is different from that of bone marrow derived MSC [19].

Because MSC do not produce IL-2 or IL-15, these cytokines remain essential for any *ex vivo* culture unless the MSC are genetically engineered to produce those cytokines. We have shown before that UC-MSC can easily be transfected [19], and hence, lend themselves to serve as customized feeders for a number of different human cells for expansion. First data indicate that other cells may be multifold expanded with modified MSC as feeders [31].

We observed the disappearance of UC-MSC during the 2-week culture time. It is possible that the initial irradiation of the UC-MSC did not support their survival for that length of time. Alternatively, the NK cells, because of the allogeneic reactivity between NK cells and UC-MSC, could lyse the MSC over time. We observed the disappearance of the layer usually after the first week. This observation is similar to that of Spaggiari et al. [32], who have described the lysis of allogeneic MSC by activated NK cells.

The expression of inhibitory NK receptors did not change significantly after coculture expansion. Expression of activating receptors by NK cells was also unchanged except for the acquisition of the activating receptor NKp44, which is usually expressed on cytokine-activated NK cells. The percentage of $CD56^{+}$/$CD117^{+}$ cells increased significantly ($P = .0216$), suggesting that our culture conditions supported expansion of NK precursors. Of note is that the expanded NK cells consisted mostly of $CD56^{bright}$/$CD16^{-}$ cells. This subpopulation is known to predominantly produce cytokines and to be less cytolytic than $CD56^{dim}$/$CD16^{+}$ cells. This could explain why we observed less killing of REH, Raji, and SUP-B15 target cells by expanded NK cells compared to just overnight-activated NK cells. Of note also is that NK cells from fresh, nonactivated $CD3^{dep}$ CB-MNC or from $CD3^{dep}$ CB-MNC expanded without feeder layer display negligible cytotoxicity.

Our long-term goal is to make NK cells targeted to cancer surface antigens through transfection with CARs [23,26,27]. To decrease risks for the recipient and minimize the regulatory requirements, we sought to achieve this objective through the use of nonviral vectors and electroporation. Previous work from our group had shown that the NK-92 cell line could be transfected with CAR with high efficiency using either a DNA vector or mRNA [27]. We therefore attempted to transfect NK cells with DNA plasmid or mRNA constructs for GFP. Although we used 2 different methods of transfection (electroporation and nucleofection), the transfection efficiency for NK cells, either activated overnight or expanded for 2 weeks, was consistently low, with the majority of cells dying off. Our results suggest that genetic manipulation of NK cells from CB is feasible, but that other likely vector constructs such as lentiviruses may be preferable for transfection [33]. In summary, we present here a method for optimized expansion of functional NK progenitor cells from CB after CD3 depletion by using a feeder layer of UC-MSC. Further studies will be directed to engineer UC-MSC to circumvent the need for exogenous cytokines and to improve the retargeting of NK cells.

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